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(54) **THERAPEUTIC AGENT, TREATMENT METHOD, PROPHYLACTIC AGENT, AND
PROPHYLACTIC METHOD FOR CANINE AND FELINE IMMUNOLOGICAL DISEASES**

(57) The present invention discloses a remedy, preventive agent, treatment method and preventive method for immune diseases of dogs and cats. As an active ingredient of the remedy and preventive agent, canine interleukin 12 is used.

Specifically, the present invention relates to an immune disease remedy and preventive agent of dogs and cats comprising canine interleukin 12 of a heterodimer formed by an amino acid sequence identical with or having a part of Sequence number:1 or Sequence number:11 and an amino acid sequence identical with or having a part of Sequence number:2 or Sequence number:12. Furthermore, the present invention relates to an immune disease treating method and preventive method for dogs and cats which is characterized in injecting the immune disease remedy and preventive agent of dogs and cats into a dog or cat.

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DescriptionTechnical Field

5 [0001] The present invention relates to an immune disease remedy and preventive agent for dogs and cats comprising canine interleukin 12 (hereinafter abbreviated as CaIL12) with the primary structure of its protein derived from canine genetic information, and an immune disease treatment method and preventive method for dogs and cats using the remedy or preventive agent.

10 Background Arts

[0002] Interleukin 12 is a heterodimer consisting of a protein with a molecular weight of about 40 kD (hereinafter abbreviated as P40) and a protein with a molecular weight of about 35 kD (hereinafter abbreviated as P35), and is a cytokine with such bioactivity as to activate natural killer cells and type 1 helper T cells (References 1 and 2), being
15 abbreviated as IL12.

[0003] For IL12, surprising treatment effects in mouse model experiments against tumors, infectious diseases, allergies, and the like especially by its powerful activity in boosting the cell-mediated immune response have been reported in the literatures (References 3, 4 and 5), and clinical trials of IL12 as a remedy against human cancers and human infectious diseases have already been started.

20 [0004] Dogs are known to suffer from various cancers such as mammary gland tumor, various dermatitis such as allergic dermatitis, various viral diseases such as Parvovirus infectious disease and distemper infectious disease, and the like. These diseases always take a high rank in a statistics on canine diseases. However, few remedies and preventive agents effective against these canine diseases are available at present. For example, most dogs suffering from cancers come to hospitals after their tumors have grown, and even if the tumors are ablated by surgery, they soon die after
25 the operation because of metastasis. Also for skin diseases often seen with dogs, they cannot be cured in most cases even if steroids are administered repetitively for long periods of time as a treatment. As a consequence, fast and continuously acting remedies are being demanded. It is expected that new applications will be attempted for these canine diseases now left without any effective remedy and preventive agent. The same thing can be said to feline diseases.

30 Disclosure of the Invention

[0005] In this situation, the inventor studied to clone CaIL12 cDNA, for mass-producing CaIL12 using it, and for providing a preparation containing CaIL12 as a remedy or preventive agent for dogs suffering from immune diseases, and succeeded in cloning cDNAs coding for the P40 and P35 of CaIL12 from canine cDNA, and furthermore succeeded in
35 producing cells capable of producing CaIL12 using two expression plasmids linked these cDNAs respectively to , and in producing recombinant Baculovirus containing both the genes. Thus, the inventor has established a method for simply mass-producing CaIL12, and found that if a preparation containing CaIL12 is administered to dogs suffering from diseases difficult to treat by conventional therapeutic methods or if lymphocytes isolated from the peripheral blood of a sick dog are stimulated in vitro by a preparation containing CaIL12 and returned into the body of the dog again, the disease can be surprisingly remarkably improved. Thus, the present invention has been completed. Surprisingly, it has
40 been also found out that the preparation containing CaIL12 shows a remarkable remedy and preventive effect for feline diseases.

[0006] The gist of the present invention is as follows:

- 45 (1) An immune disease remedy for dogs and cats comprising canine interleukin 12 of a heterodimer formed by an amino acid sequence identical with or having a part of SEQ ID NO:1 or SEQ ID NO:11 and an amino acid sequence identical with or having a part of SEQ ID NO:2 or SEQ ID NO:12.
- (2) An immune disease remedy for dogs and cats, stated in the above (1), wherein the immune disease is tumor, dermatitis, infectious disease or allergosis.
- 50 (3) An immune disease remedy method for dogs and cats which is characterized in injecting the immune disease remedy for dogs and cats stated in the above (2) or (2) into a dog or cat.
- (4) An immune disease preventive agent for dogs and cats comprising canine interleukin 12 of a heterodimer formed by an amino acid sequence identical with or having a part of SEQ ID NO:1 or SEQ ID NO:11 and an amino acid sequence identical with or having a part of SEQ ID NO:2 or SEQ ID NO:12.
- 55 (5) An immune disease preventive agent for dogs and cats, stated in the above (4), wherein the immune disease is tumor, dermatitis, infectious disease or allergosis.
- (6) An immune disease preventive method for dogs and cats which is characterized in injecting the immune disease preventive agent for dogs and cats stated in the above (4) or (5) into a dog or cat.

(7) A recombinant Baculovirus comprising both a DNA sequence identical with or having a part of sequence No:1 or Sequence No:11 and a DNA sequence identical with or having a part of Sequence No:2 or Sequence No:12.

(8) A method of producing canine interleukin 12 which is characterized in infecting an insect cell or larva with the recombinant Baculovirus stated in the above (7) and taking canine interleukin 12.

The Best Embodiments of the Invention

[0007] Plasmids in which DNAs coding for the two subunits of CalL12 protein of the present invention can be produced, for example, as described below. Two genes respectively coding for the two subunits showing CalL12 activity can be cloned by the polymerase chain reaction (hereafter abbreviated as PCR) of cDNAs synthesized after extracting poly(A)RNA from canine cells, using primers based on the gene sequences respectively coding for the two subunits of bovine or human IL12. As another method, the total length of CalL12 P40 cDNA and CalL12 P35 cDNA can be cloned by plaque hybridization of a phage library prepared from a synthesized cDNA recombinant, with two cDNA fragments obtained by PCR.

[0008] For obtaining RNA from a canine organ or cells, for example, canine monocytes or lymphocytes stimulated by a mitogen, etc., usual methods can be used, such as isolating polysomes, or using cane sugar density gradient centrifugation or electrophoresis. RNA can be extracted from said canine organ or cells, by any proper method selected from the guanidine thiocyanate cesium chloride method to effect CsCl density gradient centrifugation after guanidine thiocyanate treatment (Reference 3), phenol extraction after treatment by a surfactant in the presence of ribonuclease inhibitor using a vanadium composite (Reference 4), guanidine thiocyanate hot phenol method, guanidine thiocyanate guanidine hydrochloric acid method, guanidine thiocyanate phenol chloroform method, precipitation of RNA by treatment with lithium chloride after treatment with guanidine thiocyanate, etc.

[0009] From a canine organ or canine monocytes or lymphocytes stimulated with a mitogen, etc., mRNA is isolated by any ordinary method such as lithium chloride urea method, guanidine isocyanate method, or oligo dT cellulose column method, etc., and from the obtained mRNA, a cDNA is synthesized by any ordinary method such as Gubler et al.'s method (Reference 5) or H. Okayama et al.'s method (Reference 6), etc. For synthesizing a cDNA from the obtained mRNA, a reverse transcriptase such as avian myeloblastosis virus (AMV) is basically used, as required in combination with DNA polymerase, etc. using a primer. However, it is convenient to use a marketed synthesizing or cloning kit.

[0010] If PCR is effected with cDNAs as templates using a primer based on a human, mouse or bovine base sequence, cDNAs coding for the P40 subunit and P35 subunit showing CalL12 activity can be cloned. As another method, synthesized cDNAs are ligated to λ phage vectors, and mixed in vitro with a λ phage coated protein, etc., for packaging. The produced phage particles are infected into Escherichia coli acting as a host. In this case, the Escherichia coli infected with a λ phage is bacteriolysed, and individual clones are collected as plaques. The plaques are transferred onto a filter of nitrocellulose, etc., and by hybridization using the radio labeled genes obtained by PCR as a probe, the total length of CalL12 P40 cDNA and CalL12 P35 cDNA can be cloned.

[0011] As a host, a procaryote or eucaryote can be used. The procaryotes which can be used here include bacteria, especially Escherichia coli, Bacillus like Bacillus subtilis. The eucaryotes which can be used here include eucaryotic microbes such as yeasts, for example, Saccharomyces like Saccharomyces cerevisiae, insect cells such as Spodoptera frugiperda cells, Trichoplusiani cells and Bombyx mori cells, and animal cells such as human cells, simian cells and mouse cells. In the present invention, organisms themselves, for examples, insects such as Trichoplusiani can also be used.

[0012] The expression vectors which can be used here include plasmids, phages, phagemids, viruses (Baculovirus) (insects), vaccinia (animal cells)), etc. The promoter in the expression vector is selected, depending on the host cells. For example, promoters for bacteria include lac promoter, trp promoter, etc., and promoters for yeasts include adh1 promoter, ptk promoter, etc. Promoters for insects include Baculovirus polyhedrin promoter, p10 promoter, etc. Promoters for animal cells include early or late promoter of Simian Virus 40, etc. However, the promoters which can be used are not limited to the above.

[0013] The transformation of a host by an expression vector can be effected by any of conventional methods well known to the persons skilled in the art, and these methods are stated, for example, in Current Protocols in Molecular Biology, John Wiley & Sons. The culture of transformants can also be effected according to conventional methods.

[0014] The produced CalL12 has an apparent molecular weight of about 70 to 80 kD, if determined by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions.

[0015] The 70 ~ 80 kD band in SDS-PAGE produces two subunits of about 40 kD and about 35 kD in molecular weight under reducing conditions.

[0016] CalL12 is, as is shown in the Example 2 mentioned below, mainly characterized by the ability in vitro to induce canine interferon γ (hereinafter abbreviated as IFN γ) from canine leukocytes and the effect of promoting the proliferation of canine lymphocytes stimulated with various mitogens such as phytohemagglutinin (hereinafter abbreviated as PHA). It also has activity to activate NK cells and cytotoxic T cells for killing their target cells, for example, the cell line derived

from a tumor or fibroblasts infected with a virus.

[0017] Process for isolation and purification of CalL12 produced by a recombinant DNA technique is not limited. Conventional purification process for protein can be used. For example, relying on canine IFN γ inducing activity, purification and isolation can be carried out by combining a chromatography using ion exchange carrier, dye carrier, gel filtration carrier, silica gel carrier, chelate carrier and the like with desalting and concentration by ultrafiltration, gel filtration, dialysis, salting-out and the like.

[0018] The immune disease remedy and preventive agent for dogs and cats of the present invention show a surprising remarkable treatment effect and preventive effect against various immune diseases of dogs and cats such as diseases declining in immunological competence such as tumors, dermatitis, allergic diseases and infectious diseases, and diseases showing partial immune reaction mainly relying on liquid humoral reaction rather than on cellular immune reaction, compared to conventional remedies and preventives and treatment methods and preventive methods against these canine diseases.

[0019] In the present invention, the tumors of dogs and cats include mammary gland tumor, eosinophilic granuloma, epidermoid, ecchyma, lipoma, othematoma, pulmonary edema, dermal caulescent soft tumor, anal tumor, etc. The dermatitis of dogs and cats includes external acoustic meatus inflammation, dermatitis, eczema, dermatomycosis, pyoderma, allergic dermatitis, urtication, traumatic dermatitis, and alopecia. The infectious diseases of dogs and cats include canine Parvovirus infected disease, distemper infected disease, feline AIDS and feline leukemia, etc. Allergic diseases include canine and feline pollinosis.

[0020] The immune disease remedy and preventive agent for dogs and cats can also contain other arbitrary ingredients in addition to CalL12. The ingredients added to these medicines are mainly decided in reference to the medicine administration method. When the medicine is used as a solid, a filler such as lactose, binder such as carboxymethyl cellulose or gelatin, colorant and coating agent, etc. can be used, and such a formulation is suitable for oral administration. Furthermore, white vaseline, cellulose derivative, surfactant, polyethylene glycol, silicone or olive oil, etc. can be added as a solid or activator, to prepare a cream, emulsion or lotion, etc. for application to the diseased part as an external medicine. Moreover, when the medicine is administered as a liquid, it can contain a physiologically permissible solvent, emulsifier and stabilizer as usually practiced. The solvent can be water or isotonic physiological salt solution such as PBS, and the emulsifier can be a polyoxyethylene based surfactant, fatty acid based surfactant or silicone, etc. The stabilizer can be canine serum albumin, polyol such as gelatin or saccharide such as sorbitol or trehalose, etc. The method for administering the remedy and preventive of the present invention is not especially limited, but injection is expected to give the highest treatment effect. The injection method is not limited to intravenous injection, intramuscular injection, hypodermic injection, intraperitoneal injection or intrapleural injection. The dosage is decided in reference to the size of the solid, administration method, disease concerned, symptom, etc., and an amount enough to manifest the treatment effect and preventive effect can be administered. For example, administration of CalL12 by 0.1 pg to 100 μ g/kg per day can provide a sufficient effect.

[0021] In the case of adoptive immunotherapy, if lymphocytes isolated from 1 to 100 ml of canine or feline blood are stimulated by 0.001 pg to 1 μ g of CalL12 for 12 hours to 6 days and returned into the body again, a sufficient effect can be obtained.

Examples

[0022] The present invention is described below more concretely in reference to examples, but is not limited thereto or thereby.

[Example 1]

Cloning of CalL12 P40 and P35 genes:

(1) Preparation of canine cDNA

[0023] Total RNAs were extracted using ISOGEN (produced by Nippon Gene) from a canine liver, monocytes of the canine peripheral blood stimulated with LPS (50 μ g/ml) for 48 hours and lymphocytes derived from a canine spleen treated by avian Newcastle disease virus for 7 hours (10^7 pfu/ml). Each of the obtained RNAs was dissolved into 10 mM Tris hydrochloric acid buffer (pH 7.5) (hereinafter abbreviated as TE) containing 1 mM EDTA, and the solution was treated at 70°C for 5 minutes. Then, the same amount of TE containing 1M LiCl was added. The RNA solution was applied to an oligo dT cellulose column equilibrated by TE containing 0.5M LiCl, and washed by the same buffer. Furthermore, it was washed by TE containing 0.3M LiCl, and poly(A)RNA adsorbed by 2 mM EDTA (pH 7.0) containing 0.01% SDS was eluted. The poly(A)RNA thus obtained was used to synthesize a single stranded cDNA. That is, 5 μ g of the poly(A)RNA and 0.5 μ g of an oligo dT primer (12-18 mer) were supplied into a sterilized 0.5 ml micro centrifuga-

tion tube, and diethyl pyrocarbonate treated sterilized water was added, to make a total volume of 12 μ l. The mixture was incubated at 70°C for 10 minutes, and immersed in ice for 1 minute. To the mixture, 200 mM Tris hydrochloric acid (pH 8.4), 2 μ l of 500 mM KCl solution, 2 μ l of 25 mM $MgCl_2$, 1 μ l of 10 mM each dNTP and 2 μ l of 0.1M DTT were added, and the mixture was incubated at 42°C for 5 minutes. Then, 1 μ l of 200-unit SuperScript II RT produced by GibcoBRL was added, and the mixture was incubated at 42°C for 50 minutes, for cDNA synthesizing reaction. The reaction mixture was further incubated at 70°C for 15 minutes, and after the reaction was terminated, the reaction solution was placed on ice for 5 minutes. To the reaction solution, 1 μ l of *E. coli* RNaseH (2 units/ml) was added, and the mixture was incubated at 37°C for 20 minutes.

10 (2) Preparation of canine cDNA phage library

[0024] One microgram each of the poly(A)RNA obtained in the above (1) was used, to synthesize a double stranded cDNA using an oligo dT primer by TimeSaver cDNA Synthesis kit produced by Pharmacia according to the manufacturer's manual, and furthermore, EcoRI/NotI adaptor was ligated. Using it, cDNA Rapid Cloning Module λ gt10 produced by Amasham was used according to the manufacturer's manual, to produce recombinant λ gt10 vector, and furthermore, In Vitro Packaging Module produced by Amasham was used according to the manufacturer's manual, to produce a recombinant phage.

20 (3) Cloning of CaIL12 P40 cDNA

[0025] The following two primers:

5'ATGTGTCACCGAGTCAGTTGGTCATCTCTTGGTTT3' (sequence number 3), and

5'CTAACTGCAGGGCACAGATGCCCA3' (sequence number 4)

were synthesized by a DNA synthesizer based on the base sequences at the N-terminus and C-terminus of human IL12 P40 (Reference 1). The cDNAs obtained from a canine liver and the LPS stimulated canine peripheral blood in the above (1) were taken by 2 μ l each into different 0.5 ml micro centrifugation tubes, and respective reagents were added to contain 20 pmol of either of the primers, 20 mM Tris hydrochloric acid buffer (pH 8.0), 1.5 mM $MgCl_2$, 25 mM KCl, 100 μ M/ml gelatin, 50 μ M each dNTP and 4-unit TaqDNA polymerase, for achieving a total volume of 100 μ l in each tube. The reaction was subjected to 35 amplification cycles on a DNA Thermal Cycler produced by Perkin-Elmer Cetus. The amplification cycle profile was 94°C denaturation for 1 minute, 55°C primer anneal for 2 minutes, and primer extension at 72°C for 3 minutes. The PCR product was separated by electrophoresis in a 1% agarose gel, and an about 990 bp DNA fragment was prepared according to a conventional method (Reference 7).

[0026] The DNA fragment was ligated to T-Vector produced by Invitrogen using DNA Ligation Kit Ver. 1 produced by Takara Shuzo Co., Ltd. Using it, *E. coli* was transformed according to a conventional method, and from the obtained transformant, a plasmid DNA was prepared according to a conventional method. It was confirmed by PCR under the same conditions as mentioned before that the plasmid had a PCR fragment inserted, and the base sequence of the P40 subunit cDNA of the two subunits considered to show CaIL12 activity by the dyedeoxy method (Reference 9) was determined using Genesis 2000 DNA analysis system (produced by Du Pont). The sequence is shown as sequence number 1.

[0027] A 990 bp DNA fragment containing this sequence was labeled with ^{32}P using Random Primer DNA Labeling Kit produced by Takara Shuzo Co., Ltd., to prepare a probe. The recombinant phage library prepared from the canine liver cDNA obtained in the above (2) was formed as a plane on *E. coli* NM514, and transferred onto Hybond-N+ produced by Amasham according to a conventional method. The Hybond-N+ was incubated in 5xSSPE (0.9 M NaCl, 50 mM NaH_2PO_4 , 5 mM EDTA, pH 7.4), 5xDenhardt's solution (0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin), 0.1% SDS, 100 μ g/ml salmon sperm DNA at 65°C for 2 hours, and hybridized with 1×10^6 cpm/ml of the labeled probe prepared as described above. After overnight incubation at 65°C, the Hybond-N+ was washed in 0.2xSSC (30 mM NaCl, 3 mM sodium citrate), 0.1% SDS for 15 minutes three times, and exposed to Fuji Imaging Plate produced by Fuji Photo Film Co., Ltd. for 12 hours, being analyzed by Bioimaging Analyzer produced by Fuji Photo Film Co., Ltd. The plaques with a positive signal were re-screened according to a conventional method. As a result of three times of screening, one recombinant phage with a positive signal was obtained. From the recombinant phage, a phage DNA was extracted according to a conventional method and cleaved by restriction enzyme EcoRI. By 1% agarose gel electrophoresis, a DNA fragment of about 1.5kb was obtained, and ligated to pUC118BAP treated DNA (EcoRI/BAP) produced by Takara Shuzo Co., Ltd. using DNA Ligation Kit Ver. 2 produced by Takara Shuzo Co., Ltd. Using it, a plasmid DNA was prepared according to a conventional method, and the base sequence of the obtained DNA fragment was determined using a fluorescent DNA sequencer (DNA Sequencer 373S produced by Perkin-Elmer) and Dye Terminator Cycle Sequencing Kit produced by Perkin-Elmer according to the manufacturer's manual. Of the sequence, the sequence coding for CaIL12 P40 cDNA is shown as sequence number 11.

(4) Cloning of CaIL12 P35 cDNA

[0028] The following two primers:

5'AGCATGTGTCCAGCGCGCAGCCTCCTTGTGCTACCCTG3' (sequence number 5) and

5'CTAGGAAGAACTCAGATAGCTCATCATTCTGTCGATGGT3' (sequence number 6)

were synthesized by a DNA synthesizer based on the base sequences at the N-terminus of human IL12 P35 (Reference 1) and the C-terminus of bovine IL12 P35. An about 670 bp DNA fragment was obtained as described in the above (3), using the cDNA obtained from lymphocytes derived from a canine spleen treated by avian Newcastle disease virus of the above (1) as a template, and inserted into T-Vector, and the base sequence of the P35 subunit cDNA out of the two subunits considered to show CaIL12 activity was determined. The sequence is shown as sequence number 2. Furthermore, an about 670 bp DNA fragment containing this sequence was used to prepare a radio labeled probe. The recombinant phage library prepared from the cDNA obtained from lymphocytes derived from a canine spleen treated by avian Newcastle disease virus obtained in the above (2) was hybridized with the labeled probe as described in the above (3), and the hybrid was screened. From one recombinant phage with a positive signal obtained as a result, a DNA was extracted and cleaved by restriction enzyme NotI, and by 1% agarose gel electrophoresis, an about 1.2 kb DNA fragment was obtained. It was ligated to the NotI site of pBluescriptII produced by STRATAGENE according to a conventional method. It was used to prepare a plasmid DNA, and the base sequence of the obtained DNA fragment was determined using a fluorescent DNA sequencer. Of the sequence, the sequence coding for CaIL12 P35 cDNA is shown as sequence number 12.

[Example 2]

Production of CaIL12:

(1) Preparation of CaIL12 expression vector

[0029] Expression vector pCDL-SR α 296 (Reference 9) was cleaved with restriction enzyme EcoRI, and the terminal was dephosphorylated by an alkaline phosphatase derived from a bacterium. By 1% agarose gel electrophoresis of it, an about 3.6 kb DNA fragment was prepared according to a conventional method. On the other hand, as the CaIL12 P40 cDNA fragment, an about 990 bp DNA fragment was prepared by preparing the following two primers with the EcoRI cleaved region added:

5'GGGGAATTCATGTGTCCAGCAGTTGGTCATCTCTTGG3' (sequence number 7) and

5'CCCGAATTCCTAACTGCAGGGCACACATGCCAGTCGCT3' (sequence number 8),

performing PCR by 30 cycles using the P40 subunit DNA out of the two subunits considered to show CaIL12 activity inserted in T-Vector prepared in Example 1 (2) as a template, by denaturing the DNA at 94°C for 1 minute, annealing the primer at 55°C for 2 minutes and extending the primer at 72°C for 3 minutes, to precipitate ethanol, cleaving by restriction enzyme EcoRI and effecting 1% agarose gel electrophoresis. Furthermore, an about 990 bp DNA fragment was prepared by preparing the following two primers with the EcoRI cleaved region added:

5'GGGGAATTCATGCATCCTCAGCAGTTGGTCATCTCCTGG3' (sequence number 13) and

5'CCCGAATTCCTAACTGCAGGACACAGATGCCAGTCGCT3' (sequence number 14),

effecting PCR using the CaIL12 P40 cDNA inserted in pUC118 prepared in Example 1 (2) as a template, and cleaving by EcoRI. The obtained respective CaIL12 P40 cDNA fragments were linked to the pCDL-SR α 296 prepared as described above, using T4DNA ligase. It was used to transform E. coli, and from the obtained transformant, a plasmid DNA was prepared, to obtain FO CaIL12 P40 and FO CaIL12 P40FL expressing CaIL12 P40. Furthermore, pCDL-SR α 296 was cleaved by restriction enzyme PstI, and dephosphorylated, and electrophoresis was effected to prepare an about 3.6 kb DNA fragment. As the CaIL12P 35 DNA fragment, an about 670 bp DNA fragment was obtained by preparing the following two primers with the PstI cleaved region added:

5'GGGCTGCAGATGTGTCCAGCGCGCAGCCTCCTCTTGTG3' (sequence number 9) and

5'GGGCTGCAGCTAGGAAGAACTCAGATAGCTCATCATTCT3' (sequence number 10),

effecting PCR by 30 cycles using the P35 subunit DNA out of the two subunits considered to show CaIL12 activity inserted in T-Vector prepared in Example 1 (3) as a template, at 94°C for 1 minute, at 55°C for 2 minutes and at 72°C for 3 minutes, to precipitate ethanol, cleaving by restriction enzyme PstI, and effecting 1% agarose gel electrophoresis. Furthermore, an about 670 bp DNA fragment was prepared by preparing the following two primers with the PstI cleaved region added:

5'GGGCTGCAGATGTGCCCCGCCGCGCGCCTCCTCTTGTG3' (sequence number 15) and

5'GGGCTGCAGTTAGGAAGAACTCAGATAACTCATCATTCT3' (sequence number 16),

performing PCR using the CaIL12 P35 DNA inserted in pUC118 prepared in Example 1 (2) as a template, and cleaving by PstI. The obtained respective CaIL12 P35 DNA fragments were linked to the pCDL-SR α 296 prepared by cleaving

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by PstI using T4 DNA ligase, for transforming *E. coli* and preparing a plasmid DNA as described above, to obtain FO CalL12 P35 and FO CalL12 P35 FL expressing CalL12 P35.

[0030] The base sequences of CalL12 P40 DNA and CalL12 P35 DNA in these four expression plasmids prepared were confirmed.

(2) Production of CalL12 by COS-1 cells

[0031] Five micrograms each of the FO CalL12 P40 and FO CalL12 P35 obtained in the above (1) were added to 4 ml an ERDF medium (produced by Kyokuto Seiyaku K.K.) containing 50 mM Tris hydrochloric acid buffer (pH 7.5), 400 μ g/ml of DEAE dextran (produced by Pharmacia) and 100 μ M of chloroquine (produced by Sigma). On the other hand, the COS-1 cells (ATCC CRL-1650) grown till 50% confluent in 10% fetal bovine serum (produced by Gibco, hereinafter abbreviated as FBS) using a 10 cm dia. dish were washed by PBS once, and 4 ml of the DNA mixture obtained in the above was added. The mixture was cultured in 5% CO₂ at 37°C. Four hours later, the cells were washed by PBS, and cultured in 20 ml of ERDF medium in 5% CO₂ at 37°C for 4 days, to obtain a cultured supernatant containing produced CalL12.

(3) Preparation of recombinant Baculovirus capable of producing CalL12

[0032] To the restriction enzyme XbaI and SmaI cleaved regions downstream of the promoter of Baculovirus transfer vector pAcAB3 (produced by Pharmingen), p40 and p35 subunit cDNAs were linked respectively according to a conventional method, to obtain a recombinant transfer vector. Furthermore, recombinant Baculovirus was prepared using Baculovirus Transfection Kit produced by Pharmingen according to the attached manual.

(4) Production of CalL12 by insect cells

[0033] The recombinant Baculovirus obtained in the above (3) was infected into Sf21 cells (derived from *Spondoptera fragruda*, obtained from Pharmingen) plate-cultured till the confluent in a 75 cm² flask in BaculoGold™ Protein-Free Insect Medium produced by Pharmingen, and the infected cells were cultured for 4 days, to obtain a cultured supernatant containing produced CalL12.

(5) Activity measurement of CalL12

[0034] The activities of the CalL12 produced in the above (2) and (4) were measured as described below. To test the activity to induce canine IFN γ from canine lymphocytes, the antiviral activity and the activity to intensify the class II MHC expression of canine cells were measured.

[0035] From a canine spleen, lymphocytes were isolated, and suspended in 10% FBS-ERDF at a cell density of 10⁶ cells/ml. 2.5 ml of them and 250U of human IL2 (produced by Genzyme) was added to a 6 cm dish, and 2.5 ml of the cultured supernatant obtained in the above (2) and 250U of human IL2 (produced by Genzyme) was added to it. The mixture was cultured in 5% CO₂ at 37°C for 2 days, and the antiviral activity of the cultured supernatant was measured according to the CPE method of Reference 10 using Vesicular Stomatitis Virus as a virus and MDCK (ATCC CCL-34) as sensitive cells. As a result, an antiviral activity of more than 2 \times 10⁵ dilution units/ml was confirmed. The antiviral activity of the cultured supernatant obtained in the above (4) was also measured according to the same method mentioned above to find an antiviral activity of more than 10⁷ dilution units/ml. On the other hand, a cell culture supernatant obtained as a control by transfecting 10 μ g of pCDL-SR α 296 into COS-1 cells as in the above (2) and a cultured Sf21 cell for 3 days did not show any ability to induce the antiviral activity.

[0036] Cell strain FCB1 derived from canine mammary gland tumor tissue expressing class II MHC was used to measure the class II MHC expression intensifying activity of each of the above culture. To a 6 cm dish, 10⁵ cells of FCB1 were attached, and 5 ml of each of the above culture was added to it, for culturing in 5% CO₂ at 37°C overnight. After completion of culture, the cells were detached by trypsin, and centrifuged by a 1.5 ml micro centrifugation tube. To them, 10 μ l of rat anti-canine class II MHC monoclonal antibody (produced by Stratagene) was added, and furthermore the mixture was suspended by 50 μ l of 10% FBS-ERDF. The suspension was incubated on ice for 1 hour. It was washed by PBS and suspended by 5 μ l of FITC labeled rabbit anti-rat monoclonal antibody (produced by Serotec) and 50 μ l of 10% FBS-ERDF, and the suspension was incubated on ice for 1 hour. It was washed by PBS, and analyzed by FACScan produced by Becton Dickinson K.K. As a result, CalL12 produced from COS1 and from Sf21 increased the expression of class II MHC on FCB1 by about 20% and 60% respectively. From this, it was found that CalL12 has activity to act on canine lymphocytes for inducing canine IFN γ .

[0037] The activity to promote the proliferation of canine lymphoblasts was measured. From canine peripheral blood, lymphocytes were isolated and suspended in 10% FBS-ERDF at a cell density of 10⁶ cells/ml, and of them, 5 ml was

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added to a 6 cm dish. To it, PHA was added at a concentration of 5 µg/ml, and the mixture was cultured in 5% CO₂ at 37°C for 3 days, to make the lymphocytes blastogenic. The lymphoblasts were suspended in 10% FBS-ERDF at a cell density of 10⁶ cells/ml, and 50 µl of the suspension was added per well of a 96-well microplate. To it, the cultured supernatant obtained in the above (2) was added by 50 µl per well. As a control, 10% FBS-ERDF was added by 50 µl per well. They were cultured in 5% CO₂ at 37°C for 3 days, and the activity of CaIL12 to promote the proliferation of lymphoblasts was measured according to the MTT assay method of Reference 11. That is, 5 mg/ml of MTT (produced by Sigma) solution was added by 10 µl per well, and the mixture was cultured for further 6 hours. One hundred and fifty microliters of 0.01N isopropanol hydrochloride solution was added, and the cells were crushed ultrasonically. The absorbance at a wavelength of 595 nm was measured by a micro-plate reader (Model 13550 produced by BIORAD). As a result, the average absorbance of the control was 0.69, while the average absorbance of CaIL12 produced from COS-1 was 1.52, showing that the activity to promote the proliferation of lymphoblasts was about twice.

[0038] Furthermore, the antitumor effect of CaIL12 against canine tumors was examined. From canine peripheral blood, lymphocytes were isolated and suspended by 10% FBS-ERDF at a cell density of 5 x 10⁶ cells/ml, and of them, 5 ml was added to a 6 cm dish. To it, 500 U of recombinant human IL2 produced by Beringer-Manheim K.K. was added, and the mixture was cultured in 5% CO₂ at 37°C for 3 days. On the other hand, canine tumor cell lines FCBR1 and A72 (ATCC CRL-1542) were suspended by 10% FBS-ERDF at a cell density of 10⁵ cells/ml respectively, and the suspensions were respectively added to a 96-well plate by 50 µl per well, for adhesion to the plate. To it, 50 µl of canine lymphocytes stimulated by human IL2 were added, and furthermore 100 µl of the cultured supernatant expressing CaIL12 obtained in the above (2) or 100 µl of 10% FBS-ERDF as a control was added, and the mixture was cultured in 5% CO₂ at 37°C for 2 days. After completion of culture, the supernatant was removed completely, and MTT assay was performed. Cytotoxicity % was calculated from the following formula:

$$\text{Cytotoxicity \%} = (1 - \text{OD2}/\text{OD1}) \times 100$$

where OD1 is the absorbance of canine tumor cells cultured in a medium only, at a wavelength of 595 nm, and OD2 is the absorbance of canine tumor cells cultured with canine lymphocytes, at a wavelength of 595 nm.

[0039] As a result, in the case of FCBR1, while the control showed a cytotoxicity of 34%, CaIL12 produced from COS-1 showed a cytotoxicity of 75%. In the case of A72, while the control showed 22%, CaIL12 showed about 83%. From these, it was found that CaIL12 activates canine lymphocytes to express antitumor effect against canine tumor cells.

[Example 3]

Purification of CaIL12:

[0040] Two hundred and fifty milliliters of the cell cultured supernatant obtained in Example 2 (4) was applied to a column packed with a sulfopropyl carrier, and the column was washed by a sufficient quantity of 20 mM phosphoric acid buffer. Adsorbed fractions were obtained by elution with 0.5 ~ 1M NaCl, applied to a Blue Sepharose carrier, and washed similarly, and elution was effected with 1.1 ~ 2M NaCl, to obtain fractions. The obtained fractions were desalted by dialysis, to obtain 5ml of a purified CaIL12 fraction. According to SDS-PAGE analysis, the purity of CaIL12 in the fraction was more than 90%.

[Example 4]

Production of CaIL12 preparation:

[0041] Physiological salt solution for injection, low molecular gelatin for injection (produced by Nitta Gelatin K.K.) and sorbitol were added to the purified CaIL 12 solution obtained in Example 3, to achieve a final gelatin concentration of 0.5% and a final sorbitol concentration of 30%. Furthermore, the mixture was treated by Posidyne (produced by Pall K.K.) to remove the pyrogen, and the residue was dispensed by 1 ml each into glass vials sterilized in dry heat at 250°C for 2 hours. Subsequently, the solution was sterilely freeze-dried, to obtain a CaIL12 preparation as vials containing 1 pg to 5 µg of CaIL12 each. The CaIL12 preparation was stable at room temperature, and dissolved well into distilled water or physiological salt solution.

[Example 5]

Evaluation of medicinal effect of CaL12 preparation at cell level:

5 (1) Tumors

[0042] To see the antitumor effect of the CaL12 preparation, tumor-bearing mice were produced, and canine lymphocytes stimulated by the CaL12 preparation were injected into them to examine the tumor reducing effect. Ten 6-week-old female nude mice (BALB/C nu/nu) were purchased from Nippon Crea K.K. Canine mammary gland tumor cell line FCBR1 was transplanted by 10^8 cells subcutaneously in the back of each mouse, and about one month later, tumor-bearing mice with a tumor of 33 mm x 25 mm on the average could be produced. On the other hand, when FCBR1 was established, 10^8 cells of tumor infiltrating lymphocytes (hereinafter abbreviated as TILs) isolated according to the Whiteside et al.'s method (Reference 14) were suspended in 20 ml of 10% FBS-ERDF, and 10 ng of the CaL12 preparation prepared in Example 4 was added. The mixture was cultured in 5% CO₂ at 37°C for 2 days, to obtain TILs stimulated by CaL12 preparation. Furthermore, as a control, 10^8 cells of TILs cultured under similar conditions without adding the CaL12 preparation were obtained. These two TIL samples were injected into the tumor-bearing nude mice intravenously, five mice each, by 10^7 cells per mouse. Seven days after injection, the weight of each tumor was measured by vernier calipers, to examine the difference from the tumor weight measured before injecting each TIL. The weight of each tumor was calculated from the following formula:

$$\text{Weight of a tumor} = (\text{length} \times \text{width}^2 / 2)$$

As a result, out of five tumor-bearing mice into which the TILs stimulated by the CaL12 preparation were injected, three mice showed perfect regression of the tumor, and two mice were less than 0.2 in the relative tumor weight with the tumor weight before TIL injection as 1. On the other hand, in all the five tumor-bearing mice into which the control TILs were injected increased in tumor weight, and all the relative tumor weights were more than 1.25. From the results, it was found that the CaL12 preparation activated the TILs, expressing the tumor reducing effect.

30 (2) Allergy

[0043] To see the anti-allergic effect of the CaL12 preparation, lymphocytes derived from dogs suffering from an allergic disease were stimulated by the CaL12 preparation, to examine whether the preparation could control the expression of allergy causing factors such as IgE. From five dogs diagnosed to suffer from atopic dermatitis, 10 ml each of blood was sampled. From the respective samples, lymphocytes were immediately isolated, and to each of the lymphocyte, 10 ng of the CaL12 preparation was added in 10% FBS-ERDF in a 10 cm dish immobilized with anti-human CD3 polyclonal antibody (produced by Zenzyme), for culturing for 3 days. As a control, canine lymphocytes cultured under similar conditions without adding the CaL12 preparation were prepared. After completion of culture, some lymphocytes were collected from each dish, and a cDNA was synthesized as described in Example 1. PCR was performed using primers specific to canine IgE and canine IgE receptor cDNAs, to examine the expression of the cDNAs. As a result, the expression of the cDNAs in the canine lymphocytes cultured with the CaL12 preparation was found to be inhibited with every sample, compared to the expression achieved without adding the CaL12. From the results, it was found that the CaL12 preparation acts on canine lymphocytes, to inhibit the expression of IgE and IgE receptor which are allergy causing factors. Furthermore, according to the Fanning method (Reference 15) using anti-human CD4 polyclonal antibody (produced by Zenzyme), from the lymphocytes remaining in each dish, CD4 positive cells mainly formed by a helper T cell population were obtained. They were used to synthesize a cDNA, and PCR was performed using primers specific to CaL5 and CaIFN γ genes, to examine the expression of the cDNAs. As a result, it was found that the expression of CaL5 cDNA was inhibited by the addition of the CaL12 preparation in every case. On the other hand, the expression of CaIFN γ cDNA was intensified by the addition of the CaL12 preparation in every case. IL5 is produced from type 2 helper T cells causing humoral immune reaction such as allergic reaction, and on the other hand, IFN γ is produced from type 1 helper T cells which cause cellular immunity and inhibit humoral immunity. From the results, it was suggested that the CaL12 preparation inhibits the type 2 helper T cells in canine lymphocytes and activate type 1 helper T cells. From these results, it was found that the CaL12 preparation is promising for treating the allergic diseases of dogs.

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[Example 6]

Toxicity test of CalL12 preparation for dogs:

- 5 [0044] The toxicity of the CalL12 preparation was tested according to the following procedure. As test animals, three beagles were used.

(1) Administration method

- 10 [0045] The preparation was administered every other days 5 times in total. The dosage was gradually increased (1 ng/kg body weight at the 1st time, 5 ng/kg body weight, 25 ng/kg body weight, 250 ng/kg body weight and 1 µg/kg body weight at the 5th time). It was administered intravenously.

(2) Observation period, observation items and observation time points

- 15 [0046] The observation period was from one week before start of administration till three weeks after start of administration. The observation items included clinical symptoms (respiratory pattern, vitality, appetite, activity, visible mucous membranes, saliva, evacuation action, somnolence), body temperature, heart rate, body weight, hematological examination (hemocytometry (leukocyte count, hematocrit, thrombocyte count, hemogram), electrolyte (Na, K, Cl), bio-
20 chemical examination (BUN, Crea., GOT, GPT, CPK, Glucose, TP, Alb, Glob, A/G), urine finding, circulatory organs and automatic nervous system finding. The body weight was measured every 7 days after date of administration, and the other items were measured one week before start of administration, immediately before date of administration, 10 minutes after, 30 minutes after, 1 hour after, 1.5 hours after, 2 hours after, 4 hours after, 6 hours after, 12 hours after, 24 hours after, 48 hours after, 2 weeks after start of administration and 3 weeks after start of administration.

- 25 [0047] As a result of testing according to the above procedure, the administration of the CalL12 preparation did not show any change to be taken up as a problem. So, it has been clarified that the CalL12 preparation is not toxic to dogs at least up to the largest dosage of 1 µg/kg body weight.

[Example 7]

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Treatment and prevention of dog diseases by CalL12 preparation:

- [0048] Twelve dogs with tumors on the epidermis were injected with the CalL12 preparation locally and intravenously. Every dog had a plurality of differently sized tumors. Of the twelve dogs, eight dogs injected with the CalL12 by 10 ng -
35 1 µg per tumor locally at the tumors every 3 to 4 days 3 to 10 times in total. As a result, 90% of the tumors injected with the CalL12 completely vanished, and all the remaining tumors were reduced to less than a half each. Four dogs had tumors of more than 100 cm³ and had them metastasized to viscera such as lungs, livers and kidneys. Of the three dogs, the tumors on the epidermis were ablated by surgical operation, and immediately, the dogs were intravenously injected with the CalL12 preparation by 10 ng/kg. Subsequently, they were injected every day for 7 days at a dosage of
40 500 ng/kg. As a result, all the tumors metastasized to the viscera vanished, and recurrence was not observed at all for six months since then.

- [0049] Seven dogs diagnosed to suffer from atopic dermatitis were intravenously injected with the CalL12 preparation. These dogs were observed to have clinical symptoms such as erythema, eczema, alopecia, etc. on the skin, and much IgE was detected in the blood. In their leukocyte fractions, respective mRNAs of IL4, IL5, IL10 and IL13 were highly
45 expressed. The dogs were intravenously injected with the CalL12 preparation at a dose of 0.1 to 100 ng/kg per time every 3 days 3 to 5 times in total, and the clinical symptoms were quickly improved by one time of injection, being perfectly cured by 3 to 5 times of injection.

- [0050] Furthermore, three dogs diagnosed to suffer from pollinosis were intravenously injected with the CalL12 preparation. By administering one time at a dosage of 0.1 to 10 pg/kg, such clinical symptoms as sneeze and snivel were
50 quickly improved.

[Example 8]

Treatment of canine diseases by adoptive immunotherapy using the CalL12 preparation:

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- [0051] From five dogs and two cats diagnosed to have tumors on the epidermis and three dogs diagnosed to suffer from atopic dermatitis, 25 ml each of blood was sampled. From each of the samples, lymphocytes were isolated, and 50 U of human IL2 (produced by Zenzyme) and 100 ng of the CalL12 preparation were added in 10% FBS-ERDF in a

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10 cm dish immobilized with anti-human CD3 polyclonal antibody (produced by Zenzyme). The mixtures were cultured for 4 days. After completion of culture, lymphocytes were collected, and intravenously injected into the respective dogs and cats. As a result, all the three dogs suffering from atopic dermatitis were cured perfectly, and the dogs and cats with tumors showed a tendency of tumor reduction. The same operation was repeated every week 3 to 5 times in total, and all the tumors were perfectly regressed.

Industrial Applicability

[0052] The present invention can provide a remedy, preventive agent, treatment method and preventive method excellent against tumors, dermatitis, infectious diseases and allergic diseases of dogs and cats.

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Claims

55

1. An immune disease remedy for dogs and cats comprising canine interleukin 12 of a heterodimer formed by an amino acid sequence identical with or having a part of Sequence number:1 or Sequence number:11 and an amino acid sequence identical with or having a part of sequence number:2 or Sequence number:12.

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2. An immune disease remedy for dogs and cats according to claim 1, wherein the immune disease is tumor, dermatitis, infectious disease or allergosis.
3. An immune disease remedy for dogs and cats according to claim 2, wherein the tumor is mammary gland tumor, eosinophilic granuloma, epidermoid, ecphyma, lipoma, othematoma, pulmonary edema, dermal caulescent tumor or anal tumor.
4. An immune disease remedy for dogs and cats according to claim 2, wherein the dermatitis is external acoustic meatus inflammation, dermatitis, eczema, dermatomycosis, pyoderma, allergic dermatitis, urtication, traumatic dermatitis or alopecia.
5. An immune disease remedy for dogs and cats according to claim 2, wherein the infectious disease is canine Parvovirus infected disease, distemper infected disease, feline AIDS or feline leukemia.
6. An immune disease remedy for dogs and cats according to claim 2, wherein the allergic disease is pollinosis.
7. An immune disease remedy for dogs and cats according to any of claims 1 to 6 which is characterized in that the canine interleukin 12 of a heterodimer formed by an amino acid sequence identical with or having a part of Sequence number:1 or Sequence number:11 and an amino acid sequence identical with or having a part of Sequence number:2 or Sequence number:12 is produced by recombinant DNA technique.
8. An immune disease remedy for dogs and cats according to any of claims 1 to 6 which is characterized in that the canine interleukin 12 of a heterodimer formed by an amino acid sequence identical with or having a part of Sequence number:1 or Sequence number:11 and an amino acid sequence identical with or having a part of Sequence number:2 or Sequence number:12 is produced by using an animal cell which is transformed simultaneously by a DNA sequence identical with or having a part of Sequence number:1 or Sequence number:11 and a DNA sequence identical with or having a part of Sequence number:2 or Sequence number:12 or an insect cell or larva infected with a recombinant Baculovirus containing both a DNA sequence identical with or having a part of Sequence number:1 or Sequence number:11 and a DNA sequence identical with or having a part of Sequence number:2 or Sequence number:12.
9. An immune disease treatment method for dogs and cats which is characterized in injecting the immune disease remedy for dogs and cats according to any of claims 1 to 8 into a dog or cat.
10. An immune disease treatment method for dogs and cats according to claim 9 which is characterized in that the injection is intravenous injection, subcutaneous injection or local injection.
11. An immune disease treatment method for dogs and cats according to claim 9 or 10 which is characterized in that the dose of injection per time is 0.1 pg/kg (body weight) to 100 µg/kg (body weight).
12. An immune disease treatment method for dogs and cats which is characterized in stimulating lymphocytes isolated from canine or feline peripheral blood by the immune disease remedy for dogs and cats stated in any of claims 1 to 8 and returning them into the body of dog or cat.
13. An immune disease preventive agent for dogs and cats comprising canine interleukin 12 of a heterodimer formed by an amino acid sequence identical with or having a part of Sequence number:1 or Sequence number:11 and an amino acid sequence identical with or having a part of Sequence number:2 or Sequence number:12.
14. An immune disease preventive agent for dogs and cats according to claim 13, wherein the immune disease is tumor, dermatitis, infectious disease or allergosis.
15. An immune disease preventive agent for dogs and cats according to claim 14, wherein the tumor is mammary gland tumor, eosinophilic granuloma, epidermoid, ecphyma, lipoma, othematoma, pulmonary edema, dermal caulescent soft tumor or anal tumor.
16. An immune disease preventive agent for dogs and cats according to claim 14, wherein the dermatitis is external acoustic meatus inflammation, dermatitis, eczema, dermatomycosis, pyoderma, allergic dermatitis, urtication, traumatic dermatitis or alopecia.

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17. An immune disease preventive agent for dogs and cats according to claim 14, wherein the infectious disease is canine Parvovirus infected disease, distemper infected disease, feline AIDS or feline leukemia.
18. An immune disease preventive agent for dogs and cats according to claim 14, wherein the allergic disease is pollinosis.
19. An immune disease preventive agent for dogs and cats according to any of claims 13 to 18 which is characterized in that the canine interleukin 12 of a heterodimer formed by an amino acid sequence identical with or having a part of Sequence number:1 or Sequence number:11 and an amino acid sequence identical with or having a part of Sequence number:2 or Sequence number:12 is produced by recombinant DNA technique.
20. An immune disease preventive agent for dogs and cats according to any of claims 13 to 18 which is characterized in that the canine interleukin 12 of a heterodimer formed by an amino acid sequence identical with or having a part of Sequence number:1 or Sequence number:11 and an amino acid sequence identical with or having a part of Sequence number:2 or Sequence number:12 is produced by using an animal cell which is transformed simultaneously by a DNA sequence identical with or having a part of Sequence number:1 or Sequence number:11 and a DNA sequence identical with or having a part of Sequence number:2 or Sequence number:12 or an insect cell or larva infected with a recombinant Baculovirus containing both a DNA sequence identical with or having a part of Sequence number:1 or Sequence number:11 and a DNA sequence identical with or having a part of Sequence number:2 or Sequence number:12.
21. An immune disease preventive method for dogs and cats which is characterized in injecting the immune disease preventive agent for dogs and cats according to any of claims 13 to 20 into a dog or cat.
22. An immune disease preventive method for dogs and cats according to claim 21 which is characterized in that the injection is intravenous injection, subcutaneous injection or local injection.
23. An immune disease preventive method for dogs and cats according to claim 21 or 22 which is characterized in that the dose of injection per time is 0.1 pg/kg (body weight) to 100 µg/kg (body weight).
24. An immune disease preventive method for dogs and cats which is characterized in stimulating lymphocytes isolated from canine or feline peripheral blood by the immune disease preventive agent for dogs and cats stated in any of claims 13 to 20 and returning them into the body of dog or cat.
25. A recombinant Baculovirus comprising both a DNA sequence identical with or having a part of Sequence number:1 or Sequence number:11 and a DNA sequence identical with or having a part of Sequence number:2 or Sequence number:12.
26. A method of producing canine interleukin 12 which is characterized in infecting an insect cell or larva with the recombinant Baculovirus of claim 25 and taking canine interleukin 12.

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP98/02031

A. CLASSIFICATION OF SUBJECT MATTER

Int.Cl.⁶ A61K38/20, C12N7/01, C12P21/02 (C12P21/02, C12R1:92)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Int.Cl.⁶ A61K38/20, C12N7/01, C12P21/02

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
CAPLUS (STN), REGISTRY (STN), MEDLINE (STN), WPIDS (STN), GENESEQ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO, 96/24676, A1 (Whitehead Institute for Biomedical Research), August 15, 1996 (15. 08. 96) & EP, 815245, A1	1-26
Y	JP, 6-501009, A (Genetics Institute, Inc.), February 3, 1994 (03. 02. 94) & WO, 92/5256, A & EP, 549711, A1 & US, 5457038, A	1-26
Y	JP, 7-53594, A (F. HOFFMANN-LA ROCH AKTIENGESELLSCHAFT), February 28, 1995 (28. 02. 95) & EP, 640689, A2 & CA, 2125763, A	1-26
Y	Virgil E.C.J. Schijns, "Molecular cloning of cat Interleukin-12", Immunogenetics, Vol. 45, No. 6 (1997) P.462-463	1-26

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	"I" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier document but published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search
August 11, 1998 (11. 08. 98)

Date of mailing of the international search report
August 18, 1998 (18. 08. 98)

Name and mailing address of the ISA/
Japanese Patent Office

Authorized officer

Facsimile No.

Telephone No.

Form PCT/ISA/210 (second sheet) (July 1992)

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP98/02031

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
PX	JP, 10-36397, A (Toray Industries, Inc.), February 10, 1998 (10. 02. 98) (Family: none)	1-26
PA	FUMIYOSHI OKANO, "Cloning and Expression of the cDNA for Canine Interleukin-12", J. Interferon Cytokine Res., vol. 17, No. 11 (1997) P.713-718	1-26

